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ORIGINAL ARTICLE

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Hepatoprotective Effects of Two Vitamin D3 Formulations on Letrozole-Induced Polycystic Ovary Syndrome in Female Rats: An Experimental Study

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ABSTRACT

Background: Polycystic ovary syndrome (PCOS) is a complex endocrine and metabolic disorder that disrupts the hypothalamic-pituitary-ovarian axis, leading to ovarian dysfunction and hyperandrogenism. This experimental study aimed to compare the hepatoprotective effects of micellized vitamin D3 (MVD3) and conventional vitamin D3 (CVD3) compared to metformin (MET) in a rat model of letrozole (LTZ)-induced PCOS.

Methods: An experimental study was conducted over 90 days using 30 healthy female albino rats with regular estrous cycles. Rats were randomly assigned to five groups (six per group): control, LTZ-induced PCOS, LTZ + MET (155 mg/kg), LTZ + CVD3 (1000 IU/kg), and LTZ + MVD3 (1000 IU/kg). PCOS was induced using oral LTZ (200 µg/day), and treatments were administered daily by gavage. Estrous cycle was assessed by vaginal cytology. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels were measured to evaluate hepatic function, and liver tissues were examined histopathologically after hematoxylin and eosin staining. The mean levels of liver enzymes were compared using the independent samples *t*-test, with significance set at $P < 0.05$.

Results: LTZ-induced PCOS caused significant elevations in serum ALT, AST, and ALP levels compared with controls ($P < 0.001$). MET treatment partially reduced liver enzyme levels, but the mean levels remained higher than controls. In contrast, treatment with either MVD3 or CVD3 resulted in marked normalization of ALT, AST, and ALP levels compared with controls, being comparable to controls. Histopathological analysis supported these findings, showing severe hepatic structural damage in rates with LTZ-induced PCOS, partial improvement with MET, and restoration of normal hepatic architecture with MVD3 and CVD3.

Conclusion: MVD3 or CVD3 markedly alleviate LTZ-induced hepatic injury in PCOS rats, outperforming MET in normalizing ALT, AST, and ALP levels and restoring hepatic architecture. These findings highlight vitamin D3 as a promising adjunct for managing PCOS-related liver injury, warranting further mechanistic and clinical studies.

Keywords: Polycystic ovary syndrome ▪ Hepatoprotective effect ▪ Micellized vitamin D3 ▪ Conventional vitamin D3

1. Introduction

Polycystic ovary syndrome (PCOS) affects approximately 10–13% of reproductive-aged women and is characterized by ovarian dysfunction, menstrual disturbances, and an increased risk of infertility and miscarriage.[1] It is a common and multifactorial disease associated with both endocrine and metabolic disorders and characterized by hyperandrogenism and ovarian abnormalities, resulting from a disruption in the hypothalamic-pituitary-ovarian (HPO) axis.[2] Beyond

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reproductive symptoms, PCOS is linked to various obstetric complications such as pre-eclampsia and gestational diabetes, as well as metabolic complications such as obesity, insulin resistance, type 2 diabetes, nonalcoholic fatty liver disease (NAFLD), dyslipidemia, hypertension, and cardiovascular disorders.[3]

Pathogenesis involves HPO dysfunction and metabolic abnormalities. Disrupted pulsatility of gonadotropin-releasing hormone raises luteinizing hormone (LH) and lowers follicular stimulating hormone (FSH), enhancing androgen production from the ovaries and adrenals.[4] Hyperandrogenism impairs follicular maturation and ovulation. Estrogen levels rise due to peripheral aromatization, while progesterone declines, increasing endometrial dysfunction risk. Central features, insulin resistance and hyperinsulinemia worsen metabolic and reproductive symptoms, contributing to oxidative stress and chronic inflammation.[4]

Diagnosis relies on criteria established by the National Institutes of Health (NIH) and Rotterdam Consensus, which recognize four PCOS phenotypes based on hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology.[5] On the other hand, PCOS management is symptom-specific, using combined oral contraceptives to regulate hormones and protect the endometrium, anti-androgens for acne and hirsutism, and 5α-reductase inhibitors for alopecia.[6]

Vitamin D deficiency (<20 ng/mL), affects 67–85% of women with PCOS.[7] Vitamin D is obtained through diet and synthesized in the skin via UV exposure, then activated through hepatic and renal hydroxylation.[8] On the other hand, NAFLD, which is defined as ≥5% hepatic fat accumulation without alcohol intake,[9] is four times more prevalent in women with PCOS.[10] PCOS and NAFLD are endocrine and hepatic manifestations of metabolic syndrome, and both are driven by insulin resistance and obesity. This dysregulation increases free fatty acid release and hepatic triglyceride synthesis, promoting lipid imbalance and cardiovascular risk.[11] Letrozole (LTZ) may exert mild hepatotoxicity through toxic or immunogenic metabolites, although the mechanism remains unclear.[12] Its prolonged use may cause liver injuries,[13] leading to increased levels of liver enzymes as a result of hepatocellular damage.[14]

The current study aimed to compare the hepatoprotective effects of conventional vitamin D3 (CVD3) and micellized vitamin D3 (MVD3) on LTZ-induced PCOS in female rats, through evaluating changes in biochemical function indices and histological features of liver.

2. Methods

2.1. Study design, animals and drugs/chemicals

An experimental study was conducted at the Dissection Hall of the Faculty of Veterinary Medicine, Sana'a University, Sana'a, Yemen, over a period of 90 days. Thirty healthy female Albino rats (158.0 ± 4.2 g) were obtained from the Faculty of Veterinary Medicine at Sana'a University and acclimatized for two weeks under controlled conditions (12:12 light/dark cycle, 20.4±1.4°C, 41.2±3.9% humidity) with free access to food and water. During the second week, daily vaginal smears (11 am to 1 pm) were conducted to select rats with regular estrous cycles.[15]

MVD3 1200 IU drop solution (Micellized D3™, Numedica, Tulsa, United State) was purchased from Amazon. CVD3 film-coated tablets (Ultra® Vitamin D 4000 IU, Vitabiotics, UK), LTZ 2.5 mg film-coated tablets (Femara®, Novartis Pharmaceuticals, Switzerland), and metformin (MET) 500 mg film-coated tablets (Glucophage®, Merk Sante S.A.S, France) were commercially purchased from Sana'a.

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2.2. Experimental design

Rats were randomly allocated into five groups (six rats per group): Group I (normal control) received 1% carboxymethylcellulose (CMC) orally; Group II (LTZ-induced PCOS) received LTZ (200 µg/day) suspended in CMC; Group III (LTZ + MET) received MET (155 mg/kg) in combination with LTZ (200 µg/day);[16] Group IV (LTZ + CVD3) received CVD3 (1000 IU/kg) together with LTZ (200 µg/day);[17] and Group V (LTZ + MVD3) received MVD3 (1000 IU/kg) in combination with LTZ (200 µg/day). LTZ and all treatments were administered once daily by oral gavage for 90 days, with vitamin D3 dosing adapted from a previous study.[18] On day 90, following overnight fasting, blood samples were collected under chloroform anesthesia via cardiac puncture. Animals were then sacrificed by decapitation, and liver tissues were excised and preserved in 10% formalin for histopathological analysis.

2.3. Evaluation of estrous cycle

Vaginal cytology was performed during acclimatization period between 11 am and 1 pm, following a previously published protocol.[14] Vaginal smears were obtained by lavage using sterile normal saline, air-dried, and stained with Giemsa. Cell types were used to identify distinct cycle phases as follows: proestrus (nucleated epithelial cells), estrus (cornified squamous cells), metestrus (mixed cells with leukocyte predominance) and diestrus (abundant leukocytes).[21]

2.4. Biochemical analysis

Serum liver enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), were measured to assess the severity of hepatic injury. Analyses were performed using the Cobas c 311 automated analyzer (Roche Diagnostics, Switzerland) at the National Center of Public Health Laboratories in Sana'a.

2.5. Histopathological study

Liver tissues were fixed in 10% formalin for 24 h, processed for paraffin embedding as previously described,[17] sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E) and microscopically examined according to standard procedures.[20]

2.6. Data analysis

Data were analyzed using GraphPad Prism software, version 8.4 (GraphPad Software, Boston, MA, USA) and were presented as mean ± standard deviation (SD). The mean levels of liver enzymes were compared using the independent samples *t*-test. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of vitamin D3 and MET on serum ALT

Table 1 shows that LTZ significantly increased the mean serum ALT level (U/L) compared with the control group (66.7±9.8 *vs.* 31.7±4.0; *P* <0.001). Compared to LTZ, administration of MET significantly reduced the mean ALT level to 51.3±5.7 U/L (*P* = 0.001), although values remained higher than those of the control group. LTZ *plus* MVD3 and LTZ *plus* CVD3 resulted in further significant reductions in the mean ALT levels (32.8±2.2 U/L and 32.3±2.2 U/L, respectively; *P* <0.001) compared with LTZ alone.

Table 1: Effect of vitamin D3 formulations and MET on serum ALT level in female rats with experimentally LTZ-induced PCOS

Groups	ALT (U/L)		Adjusted
	$Mean_1$±SD	$Mean_2$±SD	*P*-value
Control *vs* LTZ	31.7±4.0	66.7±9.8	<0.001
LTZ *vs* (LTZ+MET)	66.7±9.8	51.3±5.7	0.001
LTZ *vs* (LTZ+MVD3)	66.7±9.8	32.8±2.2	<0.001
LTZ *vs* (LTZ+CVD3)	66.7±9.8	32.3±2.2	<0.001

MET, metformin; ALT, alanine aminotransferase; LTZ, letrozole; PCOS, polycystic ovary syndrome; SD, standard deviation; MVD3, micellized vitamin D3; CVD3, conventional vitamin D3.

3.2. Effect of vitamin D3 and MET on serum AST

Table 2 shows that LTZ significantly increased the mean serum AST level (U/L) compared with the control group (277.8±29.8 *vs.* 143.5±28.8; *P* <0.001).

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Compared to LTZ, administration of MET significantly reduced the mean AST level to 196.7±27.6 U/L (*P* <0.001), although values remained higher than those of the control group. LTZ plus MVD3 and LTZ plus CVD3 resulted in further significant reductions in the mean AST levels (149.2±14.3 U/L and 147.7±9.1 U/L, respectively; *P* <0.001) compared with LTZ alone.

Table 2: Effect of vitamin D3 formulations and MET on serum AST level in female rats with experimentally LTZ-induced PCOS

Groups	AST (U/L)		Adjusted
	Mean$_1$±SD	Mean$_2$±SD	*P*-value
Control *vs* LTZ	143.5±28.8	277.8±29.8	<0.001
LTZ *vs* (LTZ+MET)	277.8±29.8	196.7±27.6	<0.001
LTZ *vs* (LTZ+MVD3)	277.8±29.8	149.2±14.3	<0.001
LTZ *vs* (LTZ+CVD3)	277.8±29.8	147.7±9.1	<0.001

MET, metformin; AST, aspartate aminotransferase; LTZ, letrozole; PCOS, polycystic ovary syndrome; SD, standard deviation; MVD3, micellized vitamin D3; CVD3, conventional vitamin D3.

3.3. Effect of vitamin D3 and MET on serum ALP

Table 3 shows that LTZ significantly increased the mean serum ALP level (U/L) compared with the control group (188.0±13.7 *vs.* 63.5±13.3; *P* <0.001). Compared to LTZ, administration of MET significantly reduced the mean ALP level to 132.7±18.3 U/L (*P* <0.001), although values remained higher than those of the control group. LTZ + MVD3 and LTZ + CVD3 resulted in further significant reductions in the mean ALP levels (71.67±8.6 U/L and 77.50±5.1 U/L, respectively; *P* <0.001) compared with LTZ alone.

Table 3: Effect of vitamin D3 formulations and MET on serum ALP level in female rats with experimentally LTZ-induced PCOS

Groups	ALP (U/L)		Adjusted
	Mean$_1$±SD	Mean$_2$±SD	*P*-value
Control *vs* LTZ	63.5±13.3	188.0±13.7	<0.001
LTZ *vs* (LTZ+MET)	188.0±13.7	132.7±18.3	<0.001
LTZ *vs* (LTZ+MVD3)	188.0±13.7	71.67±8.6	<0.001
LTZ *vs* (LTZ+CVD3)	188.0±13.7	77.50±5.1	<0.001

MET, metformin; ALP, alkaline phosphatase; LTZ, letrozole; PCOS, polycystic ovary syndrome; SD, standard deviation; MVD3, micellized vitamin D3; CVD3, conventional vitamin D3.

3.4. Histopathological findings

Liver sections from the control group showed radiating cords of polygonal hepatocytes with rounded, centrally located, rounded nuclei and eosinophilic cytoplasm. The hepatocyte cords were separated by hepatic sinusoids, which were lined with Kupffer cells. A normal central vein was also observed, surrounded by regularly organized hepatic cords, indicating preserved lobular architecture (Figure 1.A). In contrast, liver sections from the group with LTZ-induced PCOS displayed several histopathological alterations, including enlarged and congested portal vein surrounded by inflammatory cells, congested hepatic artery, and dilated sinusoids (Figure 1.B), as well as congested, dilated blood vessels (Figure 2.C). Additional pathological features included congested central vein with inflammatory cell infiltration and pyknotic nuclei (Figure 2.D), and areas of infiltration, hemorrhage and fatty changes (Figure 2.E & 2.F). The MET-treated group showed generally preserved hepatic structure, with reduced fatty changes, inflammation and central veins congestion (Figure 2.J & 2.H). However, groups treated with CVD3 (Figure 2.I) and MVD3 (Figure 2.K) demonstrated normal histological architecture closely similar to that of the control group.

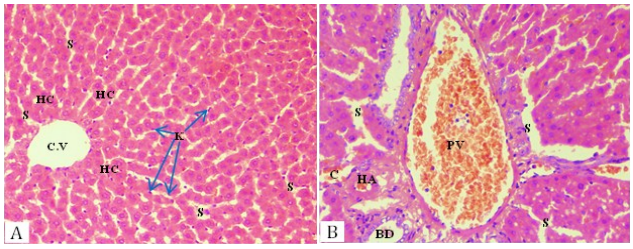

Figure 1: Photomicrograph of rat liver sections. A: Control Group displaying normal central vein (C.V), hepatocytes (HC), sinusoids (S) and Kupffer cells (K) ; B: PCOS Group: liver showing enlarged and congested portal vein (PV), congested hepatic artery (HA) and dilated sinusoids (S) (H&E x400).

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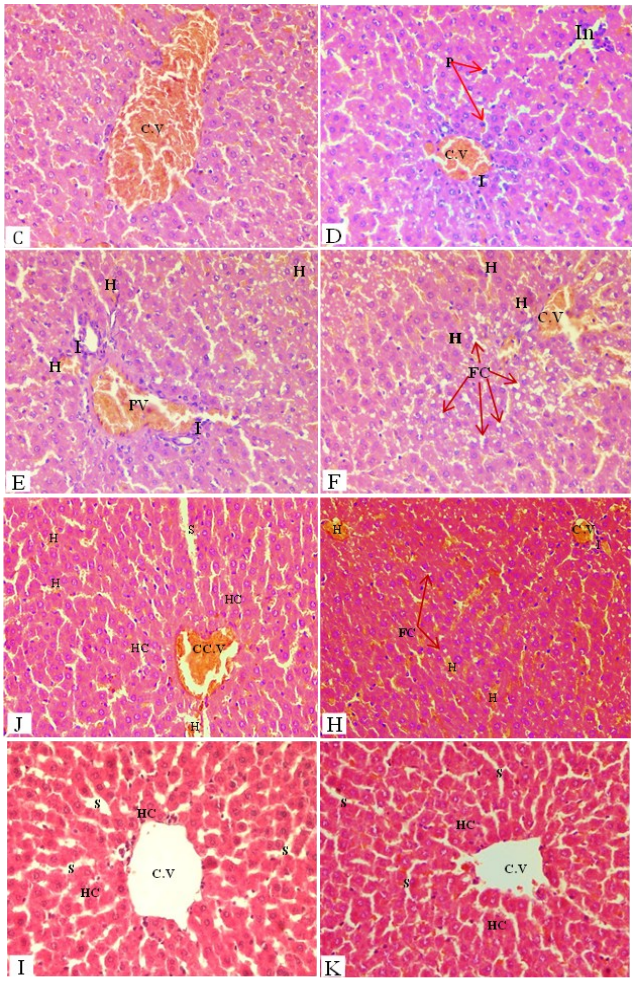

Figure 2: Photomicrograph of rat liver sections of PCOS Group. C: Liver section showing dilated and congested central vein (CV); D: Liver section showing congested central vein (CV), infiltration (I) and inflammation (In); E: Liver section showing infiltration (I) and hemorrhage (H); F: Liver section showing fatty changes (FC); J to H: MET+LTZ Group: Liver section showing generally preserved hepatic structure. I & K: CVD3 and MVD3 Groups: Liver sections showing normal structures (H&E x400).

4. Discussion

The present study assessed the efficacy of two vitamin D3 formulations (CVD3 and MVD3) in protecting hepatocytes in PCOS induced by LTZ in female rats. The PCOS in our experimental model was accompanied by hepatic dysfunction and damage, with elevated ALT, AST, and ALP levels. MET, the standard treatment of PCOS, partially ameliorated these biochemical changes. However, MVD3 and CVD3 treatments showed stronger reductions, particularly CVD3, which exhibited superior hepatoprotective effects. These findings suggest that PCOS-related liver stress can be relieved by vitamin D supplementation.

The present findings align with the results of a previous study,[21] showing significant elevations in ALT, AST, and ALP following LTZ-induced PCOS, with these alterations markedly reversed by MET treatment. However, the study design was different, employing oral LTZ at 0.5 mg/kg/day for 7 weeks, with MET (300 mg/kg/day) introduced after the first three weeks and continued thereafter.[21] Another study similarly reported a significant increase in ALP level following LTZ administration (1 mg/kg for 21 days), suggesting hepatic or biliary stress.[22] In contrast, a study found no significant changes in AST or ALT levels in rats with LTZ-induced PCOS, which may be attributed to the shorter duration of oral LTZ administration (21 days) compared with the present study.[23] Unlike the present study, another study showed no significant change in serum ALT levels and an increase only in AST and ALP following LTZ administration. This discrepancy may due to the use of Sprague-Dawley rats and LTZ in a dose of 1 mg/kg for 14 days.[13]

In the line with this study, another study reported that the estradiol valerate-induced PCOS group (4 mg/kg/day, intramuscularly for 28 days) showed a significant elevation in ALT, AST, and ALP.[24] The hepatotoxic effect of LTZ may be attributed to its suppression of estrogen synthesis. Estrogens are known for their antioxidant properties, playing a protective role against lipid peroxidation and hepatocyte apoptosis. Therefore, the estrogen-depleting action of LTZ could lead to increased oxidative stress in liver tissue, contributing to liver toxicity.[13]

Consistent with the present study, significant reductions ALT, AST, and ALP have been reported

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following MET administration, suggesting hepatoprotective effect. For instance, a previous study showed that MET (200 mg/kg/day for 28 days significantly reduced liver enzymes following PCOS induction.[11] Another study showed that MET at a lower dose (20 mg/kg for 20 days) normalized ALT levels, while AST and ALP remained comparable to controls.[25]

Younas et al[26] reported that MET (20 mg/kg for 7 weeks) significantly reduced serum AST and ALT levels in rats with LTZ-induced PCOS, while ALP levels remained unchanged. This fining partially aligns with the present findings, particularly with respect to ALT and AST reductions. On the other hand, another study showed that MET (20 mg/kg/day for 6 weeks) significantly reduced all three liver enzymes in rats with PCOS induced by LTZ (1 mg/kg/day) for 12 weeks, including six weeks of induction followed by 6 weeks of treatment.[27]

A study showed that induction of PCOS using oral LTZ (1 mg/kg for 7 weeks) resulted in significant elevations in AST and ALP levels, while ALT showed a nonsignificant decrease; however, all three liver enzymes improved significantly following MET treatment (20 mg/kg/day for 5 weeks).[28] Another study showed that induction of PCOS using oral LTZ (1 mg/kg) combined with a high-fat diet for 4 weeks resulted in significant elevations in ALT, AST, and ALP levels which were all reversed following MET treatment (200 mg/kg).[29] MET activates hepatic adenosine monophosphate activated protein kinase (AMPK), enhancing glucose uptake and reducing mitochondrial fatty acid oxidation, mechanisms that may explain its beneficial impact on liver enzymes.[30] It has been shown to exert hepatoprotective effects in PCOS-induced models by reducing serum AST, ALT, and ALP levels through alleviation of oxidative stress and restoration of liver enzyme balance, findings that are consistent with the present study despite differences in dose and treatment duration.[31]

Vitamin D supplementation (3200 IU/day for three months) has been shown to significantly reduce ALT levels in overweight and obese women with PCOS,[32] consistent with the present findings. The proposed mechanisms contributing to improved liver function include activation of hepatic vitamin D receptors, suppression of fibrotic gene expression, enhancement of autophagy, and improved insulin sensitivity.[32] Similarly, improvements in ALT levels were observed only in responders to vitamin D supplementation.[33] In contrast, a 12-week hypocaloric diet combined with 25 µg calcitriol increased ALT and AST in patients following vitamin D supplementation compared to the placebo group.[34] However, a previous study reported that daily supplementation with 4000 IU of vitamin D for 12 weeks significantly reduced serum ALT and AST levels in patients with metabolic dysfunction–associated steatotic liver disease.[35]

In the present study, PCOS induction caused marked hepatic histopathological alterations, including vascular congestion, sinusoidal dilation, inflammatory infiltration, hemorrhage, and fatty degeneration, reflecting significant oxidative and metabolic stress. MET partially alleviated these changes, whereas both CVD3 and MVD3 markedly restored normal hepatic architecture, closely resembling the control group. These histological improvements parallel the biochemical findings and highlight the superior hepatoprotective potential of vitamin D3 in mitigating PCOS-associated liver injury. In contrast to our findings, a previous study showed minimal and local changes in LTZ-treated Sprague-Dawley rats, which could be attribute to the use of LTZ in a dose of 1 mg/kg for 14 days.[13]

Our findings are consistent with reported by Abulfadle et al,[21] which demonstrated pronounced hepatic pathological changes in rats with LTZ-induced PCOS, indicating severe hepatic stress and inflammation. However, they do not fully align with

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the MET-treated group, where these structural abnormalities partially improved, possibly due to the extended duration of our study despite the relatively low MET dose administered. A study closely aligned with the present findings reported marked hepatic damage in PCOS rats, with only mild histological improvement following MET treatment, in a model where PCOS was induced using LTZ (1 mg/kg for 21 days) followed by MET administration (20 mg/kg for 7 days).[36]

Consistent with the present findings, multiple studies have demonstrated that vitamin D supplementation preserves hepatic architecture, reduces steatosis and inflammatory infiltration, promotes hepatic regeneration, and restores near-normal liver histology across different models of liver injury and dosing regimens.[20,37,38] Vitamin D exerts antioxidant effects both directly, by acting as a membrane-stabilizing antioxidant, and indirectly, by enhancing endogenous antioxidant defenses through upregulation of enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione.[39,40] It may further reduce hepatic steatosis and improve lipid metabolism by upregulating Cpt1a, a key enzyme in fatty acid β-oxidation that enhances hepatic lipid clearance, while also modulating inflammation by decreasing proinflammatory cytokines such as tumor-necrosis factor-alpha (TNF-α) and increasing the anti-inflammatory cytokine interleukin-10.[41–43] On the other hand, MET therapeutic action is largely attributed to the suppression of mitochondrial respiratory chain complex I, leading to a reduced ATP/AMP ratio and subsequent activation of AMPK, which enhances hepatic fatty acid β-oxidation while suppressing lipogenesis, thereby reducing hepatic fat accumulation.[44]

5. Conclusion

MVD3 and CVD3 significantly alleviate LTZ-induced hepatic injury in PCOS rats, surpassing MET in restoring liver enzyme profiles and histological integrity. The normalization of ALT, AST, and ALP levels, alongside preserved hepatic architecture, underscores their hepatoprotective potential. These findings offer a promising adjunctive approach to alleviate hepatic complications in PCOS management. Future studies are recommended to investigate the mechanistic basis of MVD3 hepatoprotective effects, and clinical trials are needed to validate preclinical outcomes.

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Ethical approval

The experimental protocol was approved by the Research Ethics Committee of the University of Science and Technology, Sana'a (Ethical Clerance No. 1445/0011/UREC/UST).

Conflict of Interest

The authors declare no conflict of interest associated with this article.

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